Eukaryotic elongation factor 2 controls TNF-α translation in LPS-induced hepatitis

Bárbara González-Terán, José R. Cortés, Elisa Manieri, Nuria Matesanz, Ángeles Verdugo, María E. Rodríguez, Águeda González-Rodríguez, Ángela Valverde, Pilar Martín, Roger J. Davis, and Guadalupe Sabio

Department of Vascular Biology and Inflammation, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain.
Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, Campus de Cantoblanco, Madrid, Spain.
Institute of Biomedicine Alberto Solís (CSIC/UAM), Madrid, Spain.
Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, CIBERDEM, ISCIII, Spain.
Howard Hughes Medical Institute and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

Bacterial LPS (endotoxin) has been implicated in the pathogenesis of acute liver disease through its induction of the proinflammatory cytokine TNF-α. TNF-α is a key determinant of the outcome in a well-established mouse model of acute liver failure during septic shock. One possible mechanism for regulating TNF-α expression is through the control of protein elongation during translation, which would allow rapid cell adaptation to physiological changes. However, the regulation of translational elongation is poorly understood.

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Results

MKK3 and MKK6 collaborate in LPS-induced hepatitis. To study the role of p38 MAPK signaling in hepatitis, we examined the effect of D-gal+LPS treatment on mice deficient for the upstream kinases MKK3 and MKK6. No differences in mortality were detected in Mkk3–/– or Mkk6–/– mice compared with WT animals (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI65124DS1). However, Mkk3–/– mice present less severe liver hemorrhage (Supplemental Figure 1B) and Mkk6–/– liver apoptosis was significantly reduced (Supplemental

Figure 1

ΔMKK3/6 mice are protected against LPS-induced liver damage. WT and Mkk3–/–/Mkk6–/– mice (ΔMKK3/6) were treated with D-gal+LPS or saline. (A) Survival curves after D-gal+LPS injection (n = 21). Survival curves were created with the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test. (B) Livers were removed at 6 hours after injection. Panels show representative H&E-stained liver sections and livers. The chart presents hemorrhagic area as a percentage of the total area (n = 6–8). Scale bar: 50 μm. (C) Serum transaminase activity at 4 and 6 hours after injection (n = 10). (D) Liver extracts were examined by immunoblot with antibodies to cleaved PARP, cleaved caspase 3, caspase 3, phospho-Jun and GAPDH (n = 6–8). (E) ELISA analysis of serum TNF-α and IL-6 at different times after injection (n = 16). Data are means ± SD. **P < 0.01; ***P < 0.001 (2-way ANOVA coupled to Bonferroni’s post tests).
that D-gal+LPS injection increased liver infiltration by neutrophils (Figure 2D and ref. 30). Analysis of liver cell populations revealed that p38αMkk3 Δ/– mice recently shown to control neutrophil infiltration (28), and p38αMkk3 Δ/– mice (Supplemental Figure 2A). In vitro stimulation of isolated BM macrophages from these mice with LPS also revealed no differences in proliferation rate or released TNF-α and IL-6 levels (Supplemental Figure 2, B and C). Moreover, supernatants from WT, Mkk3 Δ/–, and Mkk6 Δ/– macrophages induce apoptosis similarly in cultured hepatocytes (not shown), thus indicating that the partial protection against LPS-induced liver apoptosis was not due to circulating macrophages.

Neutrophil infiltration can cause mild to severe liver damage (28), and p38α was recently shown to control neutrophil cytokine production (29). We examined liver myeloid leukocytes based on CD11b, Gr-1, F4/80, and Ly6C markers (Supplemental Figure 2D and ref. 30). Analysis of liver cell populations revealed that D-gal+LPS injection increased liver infiltration by neutrophils (CD11b Gr-1Ly6G) in WT mice, but this effect was impaired in Mkk3 Δ/– and Mkk6 Δ/– mice (Supplemental Figure 1E). To investigate the potential involvement of liver neutrophils and macrophages in the protection detected in Mkk3 Δ/– and Mkk6 Δ/– mice, we isolated leukocytes from the livers of WT, Mkk3 Δ/–, and Mkk6 Δ/– mice and stimulated them with LPS. Whereas the percentage of neutrophils expressing TNF-α was the same in all genotypes, the percentage of monocytes (CD11bGr-1Ly6G) expressing TNF-α was higher in WT mice than in Mkk3 Δ/– and Mkk6 Δ/– mice (Supplemental Figure 1F), indicating that the protection against LPS-induced liver apoptosis could be mediated by liver inflammatory monocytes.

Mkk3 Δ/– Mkk6 Δ/– mice are protected against LPS-induced liver failure. MKK3 and MKK6 show functional redundancy in the activation of p38 MAPK (14), and while single mutants are viable, doubly deficient Mkk3 Δ/– Mkk6 Δ/– and Mkk3 Δ/– Mkk6 Δ/– mice die early in embryogenesis. To minimize interference from redundant actions, we used Mkk3 Δ/– Mkk6 Δ/– (ΔMKK3/6) mice, which are viable (14). As expected, ΔMKK3/6 mice were protected against LPS-induced hepatotoxicity, showing milder hemorrhage and better survival than WT mice (Figure 1, A and B). Immunoblot analysis demonstrated that D-gal+LPS injection activated caspase 3 and PARP cleavage in WT liver but not in ΔMKK3/6 livers (Figure 1D). Moreover, circulating transaminases were reduced in ΔMKK3/6 mice, indicating a reduction in liver necrosis (Figure 1C). These data demonstrate that MKK3/6 deficiency severely limits LPS-stimulated hepatic apoptosis. Examination of serum TNF-α after D-gal+LPS injection revealed that the marked increase in TNF-α concentration observed in WT and singly deficient mice was not detected in ΔMKK3/6 mice (Figure 1E). Serum IL-6 was also lower in ΔMKK3/6 mice (Figure 1E). The loss of LPS-stimulated TNF-α expression in ΔMKK3/6 mice might account for the finding that LPS activation of JNK pathway in the livers of WT mice was not seen in ΔMKK3/6 mice (Figure 1D).

MKK3 and MKK6 are required for neutrophil migration after LPS injection. The lower TNF-α production in ΔMKK3/6 mice could be a consequence of the lower leukocyte infiltration in liver. To address this, we analyzed myeloid cell migration after D-gal+LPS injection. Quantification of liver chemokines showed that in WT mice, LPS treatment increased mRNA expression of molecules important for migration of macrophages (MCP1 and ICAM) and neutrophils (KC, MIP2), whereas induction of these chemokines and adhesion molecules was decreased in the livers of ΔMKK3/6 mice (Figure 2A). Phenotypic characterization of liver-infiltrating leukocytes from control and D-gal+LPS–treated livers revealed a significant increase in neutrophils in the inflammatory cell infiltrate of WT mice that was impaired in ΔMKK3/6 mice (Figure 2B), consistent with the reduced disease severity (28). However, the percentages of macrophages and monocytes did not differ between genotypes (Figure 2B). These findings correlated with lower RNA expression of neutrophil markers (Elastase 2, Ly6G) in the livers of LPS-injected ΔMKK3/6 mice (Supplemental Figure 3A).

The reduced liver neutrophil infiltration in ΔMKK3/6 mice could be due either to impaired neutrophil release from BM or to a defect in cell migration. The levels of circulating neutrophils after LPS injection showed a sharp increase in WT mice that was absent in ΔMKK3/6 mice (Figure 2C). These data show that the reduced neutrophil infiltration in liver is in part caused by lowered neutrophil mobilization from the BM. To investigate whether the low circulating levels of TNF-α in ΔMKK3/6 mice might be responsible for this alteration, we injected TNF-α+D-gal into WT and ΔMKK3/6 mice, resulting in increased circulating neutrophil levels in both genotypes (Supplemental Figure 3B). These data indicate that the defect in migration is secondary to the low production of TNF-α in ΔMKK3/6 mice.

MKK3 and MKK6 regulate TNF-α production by macrophages. To investigate the molecular mechanism underlying reduced circulating TNF-α in ΔMKK3/6 mice, we treated BM-derived macrophages from WT and ΔMKK3/6 mice with LPS. Whereas treatment of WT macrophages with LPS activated JNK, ERK, and p38 MAPK, in ΔMKK3/6 macrophages the activation of p38 MAPK was impaired (Supplemental Figure 3C). The ΔMKK3/6 macrophages also secreted markedly reduced amounts of TNF-α and IL-6 compared with WT macrophages, confirming that MKK3/6 deficiency causes major defects in TNF-α production (Supplemental Figure 3D). Further analysis showed that neutrophils and Kupffer cells, 2 other myeloid cell types implicated in liver damage, also secreted reduced amounts of TNF-α and IL-6 (Supplemental Figure 3, E and F). However, in vivo analysis indicated that although the percentage of liver neutrophils expressing TNF-α after D-gal+LPS injection was the same in both genotypes, the percentage of monocytes and macrophages expressing TNF-α was higher in WT livers (Figure 2D).

To confirm that reduced cytokine production by liver macrophages is responsible for the protection against hepatocyte apoptosis in ΔMKK3/6 mice, we stimulated primary hepatocytes with conditioned medium from LPS-treated BM-derived macrophages. Conditioned medium from LPS-treated WT macrophages increased hepatocyte apoptosis, measured by cleavage of caspase 3 and PARP, whereas conditioned medium from LPS-treated ΔMKK3/6 macrophages had no effect (Supplemental Figure 3G). MKK3 and MKK6 are not required for TNF-α-induced liver damage. Our results so far show that MKK3/6 deficiency causes decreased TNF-α production in a TNF-α–dependent hepatitis model. This implies that ΔMKK3/6 mice are defective for TNF-α secretion, but not TNF-α responsiveness. To test this, we treated mice with TNF-α, finding that TNF-α caused similar mortality and liver damage in
ΔMKK3/6 mice have lower liver inflammation after LPS injection. WT and ΔMKK3/6 mice were treated with D-gal+LPS or saline. (A) Total RNA was extracted from livers 6 hours after treatment and chemokine mRNA levels determined by qRT-PCR. mRNA expression was normalized to Gapdh ($n = 5–8$). (B) Liver myeloid subsets (CD11b+Gr-1$^{hi}$, CD11b+Gr-1$^{int}$, CD11b+Gr-1$^{-}$) were assessed by flow cytometry of liver leukocytes isolated from WT and ΔMKK3/6 mice 4 and 6 hours after treatment. Representative dot plots are shown. Bar charts show each myeloid population as the percentage of total intrahepatic leukocyte population ($n = 7$). (C) Neutrophils as a percentage of circulating leukocytes, measured in total blood 4 hours after injection ($n = 5–8$). (D) TNF-α and IL-12 production by liver myeloid subsets were analyzed by intracellular staining in neutrophils (CD11b+Gr-1$^{hi}$), monocytes (CD11b+Gr-1$^{int}$), and CD11b+Gr-1$^{-}$ myeloid cells isolated from WT and ΔMKK3/6 mice 4 and 6 hours after injection. Representative dot plots are shown for all treatment groups, and bar charts show TNF-α-positive cells as the percentage of each myeloid population ($n = 7$). Data are means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (2-way ANOVA coupled to Bonferroni’s post tests).
WT and ΔMKK3/6 mice (Figure 3, A and B). Immunoblot analysis demonstrated that TNF-α stimulated a similar degree of caspase 3 and PARP cleavage (Figure 3C). Moreover, TNF-α induced the same levels of neutrophil liver infiltration in both genotypes (Figure 3D). To confirm that the protection was due to defective cytokine production and not to a protective loss of responsiveness to TNF-α, we treated primary hepatocytes from WT or ΔMKK3/6 mice with TNF-α and the protein synthesis inhibitor cycloheximide (CHX). Both hepatocyte populations presented the same level of apoptosis (Figure 3E). These data demonstrate that ΔMKK3/6 mice do not exhibit resistance to TNF-α–induced hepatitis and indicate that the primary protection against LPS-induced hepatitis in these mice is due to an inability to produce TNF-α.

MKK3 and MKK6 are required in the hematopoietic compartment for LPS-induced liver damage. The source of TNF-α in mouse models of hepatitis is the hematopoietic compartment (31, 32). Therefore, Mkk3 and Mkk6 must function in myeloid cells to protect against LPS-induced liver damage.
ΔMKK3/6 hematopoietic cells protect mice against hepatitis. Lethally irradiated WT mice were reconstituted with BM from WT or ΔMKK3/6 mice. Mice were treated with D-gal+LPS 2 months after transplantation. (A) $2 \times 10^7$ freshly prepared CD45.2 whole BM mononuclear cells were transplanted into lethally irradiated B6.SJL (CD45.1) mice, and engraftment by CD45.2 cells (%) was analyzed by antibody staining and FACS of peripheral blood after 4 months. Left panels: representative FACS dot plot of CD45.1 and CD45.2 expression in cells isolated from the blood of transplanted mice. Right panels: representative FACS dot plot of CD45.1 and CD45.2 expression in F4/80-positive cells isolated from the liver of transplanted mice ($n = 3$). (B) Survival curves after D-gal+LPS injection ($n = 10$). Survival curves were created with the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test. (C) Representative H&E-stained sections of livers extracted 6 hours after injection. The chart presents hemorrhagic area as a percentage of the total area ($n = 5–8$). Scale bar: 50 μm. (D) Serum transaminase activity at 4 and 6 hours after injection ($n = 5–8$). (E) ELISA of serum TNF-α and IL-6 at different times after injection ($n = 5–8$). Data are means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (2-way ANOVA coupled to Bonferroni’s post tests).
MKK3 and MKK6 are the main regulators of the α subunit of p38 (p38α). These kinases are activated in macrophages, neutrophils, and Kupffer cells by LPS or TNF-α, leading to the phosphorylation of its substrate, eEF2K (19). No changes in ERK phosphorylation were observed (Supplemental Figure 4A). The role of hematopoietic cells, we constructed radiation chimeras using lethally irradiated donor mice (Figure 4A). The phenotype found in p38γ/Lyz-KO mice might reflect the function of these kinases in several myeloid cell populations implicated in D-gal+LPS damage (such as neutrophils, Kupffer cells, and macrophages). Analysis of these cell types in Lyz-cemice revealed expression of p38γ and p38δ in macrophages, predominant expression of p38δ in neutrophils, and exclusive expression of p38γ in Kupffer cells (Supplemental Figure 5A). Moreover, p38γ is strongly activated in Kupffer cells by LPS or TNF-α treatment (Supplemental Figure 5B), as judged by impaired p38 phosphorylation observed by FACs in Kupffer cells from p38γ/Lyz-KO mice. Furthermore, analysis of phospho-p38 by Western blot confirmed that p38γ was the isoform activated in these cells after LPS treatment (Supplemental Figure 5C). LPS and TNF-α also activated p38δ and p38δ in macrophages (Figure 6), while in neutrophils, p38δ was the only isoform activated after either stimulus (Supplemental Figure 5B and D). Since neutrophils, Kupffer cells, and macrophages express p38γ, p38δ, or both isoforms, any of these cells populations could have a role in the protection observed in p38γ/Lyz-KO mice. We therefore analyzed the different leukocyte populations that infiltrated the liver after D-gal+LPS injection. Analysis of liver-infiltrating leukocytes showed lower numbers of neutrophils in LPS-injected p38γ/Lyz-KO mice than in similarly treated Lyz-cemice. However, this reduction was not significant in LPS-injected p38δ/Lyz-KO mice (Figure 5G). None of the myeloid-specific p38-deficient mice showed alterations in the percentages of infiltrated macrophages or monocytes. Collectively, these data show that p38γ/Lyz-KO mice are more strongly affected than either of the single conditional knockouts; however, p38δ/Lyz-KO animals have a stronger phenotype than p38γ/Lyz-KO mice, indicating partial

**Myeloid expression of p38γ and p38δ is required for LPS-induced hepatitis.** To investigate the role of p38γ and p38δ in LPS-induced hepatitis, we generated mice with myeloid cell–specific ablation of these p38 isoforms, separately and in combination. Mice carrying conditional loxP-flanked alleles for p38γ, p38δ, and p38β (p38γfl, p38δfl, and p38βfl mice) were crossed with Lyz-cemice. Immunoblot analysis confirmed ablation of the appropriate kinases in macrophages, neutrophils, and Kupffer cells (Supplemental Figure 5, A and B).

Figure 5

p38δ/Lyz-KO mice are protected against LPS-induced liver damage. p38β/Lyz-KO, p38δ/Lyz-KO and control Lyz-cemice were injected with D-gal+LPS or saline. (A) Mouse survival after D-gal+LPS injection (n = 14). Survival curves were created with the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test. (B) Livers were removed at 6 hours after injection. Panels show representative H&E-stained liver sections and livers (n = 5–8). Scale bar: 50 μm. (C) Hemorrhagic area as a percentage of the total area on H&E-stained liver sections (n = 5–8). (D) Immunoblot analysis of liver extracts (n = 5–8). (E) Serum transaminase activity at 6 hours after infection (n = 8–10). (F) ELISA of serum TNF-α and IL-6 at different times after infection (n = 10). (G) Liver myeloid subsets (CD11b+Gr-1hi, CD11b+Gr-1intermediate, CD11b+Gr-1–) were assessed by flow cytometry of liver leukocytes isolated from p38γ/Lyz-KO, p38δ/Lyz-KO, p38β/Lyz-KO, and control Lyz-cemice 6 hours after infection. Representative dot plots are shown. Bar charts show each myeloid population as a percentage of the total intrahepatic leukocyte population (mean ± SD; n = 4–6). Circulating neutrophils in total blood were measured as a percentage of circulating leukocytes 4 hours after infection (n = 5–8). Data are means ± SD. **P < 0.01; ***P < 0.001 (2-way ANOVA coupled to Bonferroni’s post tests).
Figure 6  

p38γ/δγ/δ macrophages promote TNF-α translation through phosphorylation of eEF2 kinase.  
(A–D) BM-derived macrophages from p38γ/δγ/δ and Lyzs-cre mice were treated with LPS (10 μg/ml) or TNF-α (20 ng/ml). Data were analyzed by 2-way ANOVA coupled to Bonferroni's post tests.  
(A) ELISA of TNF-α and IL-6 in supernatants.  
(B) qRT-PCR of Tnfa and Il6 mRNA normalized to Gapdh.  
(C) Immunoblot of BM-derived macrophage (BMM) lysates.  
(D) Flow cytometry of phospho-p38 in BM-derived macrophages stimulated with LPS and TNF-α for 30 minutes. Intracellular phospho-p38 is depicted in black; gray corresponds to the isotype control.  

(E–I) Cells were treated with LPS.  
Tnfa mRNA was detected by qRT-PCR in immunoprecipitates (1 mg protein) obtained with anti-eEF2. mRNA amounts are expressed relative to the amount detected in control IgG immunoprecipitates. Data were analyzed by Student's t test unless otherwise indicated.  

(E) Tnfa mRNA in RAW 264.7 cell immunoprecipitates.  
(F) Tnfa, Hif1α, and Il6 mRNA in RAW 264.7 cell immunoprecipitates.  

The presence of eEF2 was determined by immunoblot (upper panel) and quantified with ImageJ (1-way ANOVA coupled to Bonferroni's post tests).  

(G) RAW 264.7 cells were pretreated with DMSO or BIRB796 (10 μM) for 30 minutes before stimulation with LPS (60 minutes).  
Tnfa mRNA was measured in immunoprecipitates.  

(H and I) Tnfa mRNA in immunoprecipitates of BM-derived macrophages from Lyzs-cre and 38γ/δγ/δ mice (H) or WT or ΔMKK3/6 mice (I) after stimulation (60 minutes). eEF2 in immunoprecipitates was detected by immunoblot (upper panel).  

Data are means ± SD (n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

To assess whether eEF2 controls TNF-α elongation, we examined the binding of eEF2 to Tnfa mRNA in immunoprecipitates of mRNA-eEF2 complexes in RAW cells. RAW extracts were incubated with anti-eEF2 or an isotype control antibody, and the presence of Tnfa mRNA in the immunoprecipitates was examined by quantitative q real-time PCR. High relative levels of Tnfa mRNA were detected in the anti-eEF2 immunoprecipitate, and this association was increased by LPS treatment (Figure 6, E and F). High mRNA levels for Hif1α, a known eEF2 target, were also detected in the anti-eEF2 immunoprecipitate (Figure 6F). In contrast, Il6 mRNA levels did not differ between the anti-eEF2 and isotype control immunoprecipitates (Figure 6F). Pretreatment of RAW cells with BIRB796 abolished the association of eEF2 with Tnfa mRNA (Figure 6G). Moreover, Tnfa mRNA was detected in anti-eEF2 immunoprecipitates from LPS-treated Lyzs-cre or WT BM–derived macrophages but not in immunoprecipitates from similarly treated p38γ/δγ/δ KO or ΔMKK3/6 macrophages (Figure 6, H and I). Together, our data indicate that Tnfa mRNA is a selective target of eEF2 and that eEF2 controls TNF-α elongation. 

Redundancy between p38γ and p38δ, with a predominant role of p38δ. This overlapping action has been shown before and might account for the limited protection observed in single conditional knockouts (36, 37). We therefore used p38γ/δγ/δ mice for further experiments. Further studies showed that the reduced neutrophil infiltration in liver is, in part, caused by lowered neutrophil mobilization from the BM (Figure 5G), probably due to the defective production of TNF-α. Furthermore, measurement of TNF-α-stimulated chemotaxis in a chamber assay revealed a slightly lower than-control mobility in p38γ/δγ/δ neutrophils (Supplemental Figure 8A), and this effect was not increased by stimulation with a cytokine cocktail (Supplemental Figure 8B). Neutrophils produce several cytokines, and this function can be controlled by p38α (29). Examination of cytokine production in our system showed that p38γ/δγ/δ neutrophils express slightly reduced levels of TNF-α and IL-6 after LPS treatment, but not sufficient to explain the phenotype observed (Supplemental Figure 8C).

To investigate whether the lower neutrophil infiltration in the livers of p38γ/δγ/δ mice could account for the protection observed, we investigated whether neutrophil-specific depletion in Lyzs-cre mice results in milder liver damage upon D-gal+LPS treatment, similar to the phenotype of p38γ/δγ/δ mice. GR1 is highly expressed on neutrophils, and anti-GR1 antibody treatment has been shown to specifically deplete neutrophils (38). Treatment of mice with anti-GR1 one day before D-gal+LPS injection completely depleted Gr1+ neutrophils (Supplemental Figure 8D). Neutrophil depletion did not affect liver damage in Lyzs-cre mice, and neither did it abolish the protection observed in p38γ/δγ/δ, as assessed by the levels of ALT and AST, cleaved caspase 3, and PARP, or liver damage observed in H&E-stained sections (Supplemental Figure 8, E–G).

These data indicate that neutrophils do not play a dominant role in the protection against D-gal+LPS–stimulated hepatic damage observed in p38γ/δγ/δ mice and therefore indicate that a different myeloid cell type underlies this phenotype. Available evidence suggests an involvement of cytokine and chemokine production by activated Kupffer cells and infiltrated macrophages in the pathogenesis of liver damage (39). We therefore examined cytokine production by macrophages and Kupffer cells upon LPS treatment. ELISA analysis demonstrated that LPS-induced production of TNF-α and IL-6 was lower in p38γ/δγ/δ macrophages and Kupffer cells than in Lyzs-cre control cells (Figure 6A and Supplemental Figure 7C). Moreover, only the supernatant from macrophages of p38γ/δγ/δ animals was unable to induce apoptosis in primary hepatocytes (Supplemental Figure 7A), and analysis of this supernatant indicated a defect in cytokine production (Figure 6A and Supplemental Figure 7B). Similarly, induction of apoptosis by supernatant from control Lyzs-cre macrophages was blocked by addition of anti–TNF-α antibody, resulting in a protection similar to that obtained with p38γ/δγ/δ KO supernatant (Supplemental Figure 7C). This finding thus indicates that the low TNF-α levels in p38γ/δγ/δ macrophages and Kupffer cells could account for the protection against D-gal+LPS–induced liver damage.

p38γ and p38δ mediate TNF-α translation in myeloid cells by phosphorylating eEF2K. p38 MAPK can promote cytokine expression at the level of transcription, after transcription (by promoting mRNA stability), and translation (27, 40). Il6 mRNA expression was reduced in p38γ/δγ/δ macrophages, but levels of Tnfa mRNA were comparable to those in Lyzs-cre macrophages (Figure 6B). The stability of Tnfa mRNA also showed no difference between both genotypes (Supplemental Figure 7F). The translational action of p38δ has been linked to eEF2 kinase, the enzyme that inactivates eEF2. This kinase is inhibited by p38δ-mediated phosphorylation on Ser359 and also by ribosomal protein S6 kinase–mediated phosphorylation on Ser366 (19, 23). Since little is known about the regulation of eEF2K in macrophages, we investigated the effect of p38δ deletion on the phosphorylation of eEF2K and its downstream substrate eEF2. LPS stimulation of Lyzs-cre macrophages induced eEF2K phosphorylation on Ser359, but this was markedly impaired in p38γ/δγ/δK macrophages (Figure 6C). This observation was also corroborated in Kupffer cells (Supplemental Figure 7D). eEF2K phosphorylation in Lyzs-cre macrophages correlated with dephosphorylation of eEF2, implying activation of elongation. In contrast, p38γ/δγ/δ macrophages sustained high levels of eEF2 phosphorylation after the stimulus, suggesting that elongation is abolished.
the MKK3/6-p38γδ/eEF2K pathway, by releasing eEF2K-mediated inhibition of eEF2 activity, promotes the translational elongation of TNF-α in macrophages.

eEF2 controls TNF-α translation elongation. To confirm eEF2 control of TNF-α translation elongation, we used a recombinant lentivirus expressing an eEF2 shRNA (shEF2), which causes robust eEF2 knockdown in RAW cells (Supplemental Figure 9A). In macrophages, shEF2-mediated loss of eEF2 caused a decrease in TNF-α production of a magnitude similar to that caused by loss of p38γδ (Figure 7A). In contrast, p38γδ-dependent IL-6 production was eEF2 independent (Figure 7A). These data indicate that the expression of TNF-α is controlled by eEF2, while the reduced IL-6 production in p38γδ/Δα/eEF2K mice is caused by a different mechanism, most likely the loss of p38γδ/Δα-regulated Il6 gene transcription.

To test the role of eEF2 in vivo, we injected mice with the shEF2 lentivirus or control shRNA lentivirus 1 week before exposure to D-gal+LPS. Blood levels of TNF-α were strongly reduced by the shEF2-injected mice compared with controls (Figure 7B). Moreover, this reduction in TNF-α was associated with protection against hepatic cell death, measured by cleavage of caspase 3 and PARP and protection against hepatic necrosis, and measured by liver hemorrhage and blood levels of ALT and AST (Figure 7, C–E). Collectively, these data indicate that p38γδ/Δα MAPK proteins are required for the translation elongation of TNF-α in macrophages mediated by phosphorylation of eEF2K and the activation of eEF2.

Discussion

In this study, we show that protein kinases p38γδ/Δα mediate the development of LPS-induced acute hepatitis by acting within a protein kinase signaling network that regulates the production of TNF-α by hematopoietic cells. MKK3 and MKK6 activate p38γδ (14, 15), which in turn phosphorylate and inactivate eEF2 kinase (41). Once eEF2K is inactivated, eEF2 is dephosphorylated and activated, allowing the translational elongation of nascent TNF-α. To our knowledge, this is the first report that eEF2 or p38γδ/Δα controls cytokine production in myeloid cells. Our findings raise 3 key areas of interest: the specific role of myeloid cells, the involvement of p38γδ/Δα compared with that of the other kinases of the same family, and how acute inflammatory responses can be controlled through tight regulation of translational elongation.

Myeloid cells such as Kupffer cells and other macrophages are well known to be a critical source of TNF-α in LPS-induced hepatitis (14, 32). This specific role of hematopoietic cells is consistent with our finding that the protective effects of MKK3/6 deficiency are also present in irradiated WT mice reconstituted with ΔMKK3/6 BM (Figure 5). Furthermore, this protection was also observed in mice specifically lacking p38γδ/Δα in the myeloid compartment (Figure 6). Two cell types of special relevance in acute hepatitis are neutrophils and monocytes/macrophages (including Kupffer cells), and both of these BM-derived cell populations are essential for the innate immune response (42). Monocytes/macrophages bind to microbial constituents (such as LPS and cell wall constituents of Gram-positive bacteria), producing large amounts of pro- and antiinflammatory cytokines (3). Neutrophil recruitment and activation occur through TNF-α-mediated chemokine production (3). This is evidenced by our finding that low expression of TNF-α in ΔMKK3/6 or p38γδ/Δα mice results in decreased chemokine production and neutrophil migration, an effect reversed by injection with TNF-α. We did not find, however, any evidence for a role of p38γδ/Δα in neutrophil migration, and we found only a minor effect on cytokine expression. This contrasts with the recent finding that p38δα can control cytokine production by neutrophils (29), illustrating the different roles and clinical potential of p38 kinase isoforms as targets for preventing LPS-induced damage.

Previous reports have identified p38α as a key kinase involved in TNF-α production (43). p38α MAPK deficiency in macrophages results in decreased TNF-α production, with a modest effect on IL-6 expression (44). However, specific inhibition of this kinase has been shown to be hepatotoxic, hindering its clinical use. For example, the p38α inhibitor AMG 548 showed more than 85% inhibition of ex vivo LPS-induced TNF-α in healthy males, but its production and clinical use were suspended due to random liver enzyme elevations that were not dose or exposure dependent (45). Our results show that specific inhibition of p38α/β with SB203580 intensifies D-gal+LPS–induced liver damage, whereas BIRB796, which inhibits all 4 p38 isoforms (α/β/γ/δ) improves liver condition, including a reduction in apoptosis and necrosis. The toxicity associated with SB203580 might be caused by inhibition of p38α in hepatocytes, since mice with specific p38α deficiency in hepatocytes have increased JNK activity and increased susceptibility to liver damage (17, 43). In clinical use, BIRB796 also produces some liver enzyme elevations (46), most likely because of inhibition of p38α. Our findings indicate that the generation of inhibitors that specifically target p38γδ/Δα kinases might avoid the adverse effects found with p38α/β inhibitors.

Our results further show that p38γδ/Δα control macrophage production of TNF-α by promoting protein elongation during translation by eEF2. Protein synthesis is tightly regulated at transcriptional and posttranscriptional levels. Owing to the relatively long life time of mRNA transcripts, transcriptional regulation is commonly involved in slow, long-term cell responses, whereas immediate cell responses require posttranscriptional regulation of mRNA stability or translation. Two physiological situations requiring rapid regulation of protein synthesis are starvation and inflam-
In summary, we conclude that p38γ/δ in myeloid cells promote TNF-α production by activating its translation without changes in mRNA levels. This is achieved by phosphorylation of eEF2K, releasing the inhibitory action of this kinase on eEF2. This post-transcriptional regulation might be an important mechanism regulating cytokine secretion during the innate immune response and provides potential targets for the treatment of liver diseases.

Methods

Mice. Mkk3−/− (B6.129-Map2k13tm1cfe/J) (47, 48) and Mkk6−/− (B6.129-Map2k6tm5zw) mice (49) were as previously described (14). p38γ (B6.129-Mappk12tm1v) and p38δ (B6.129-Mappk1δtm1) mice were crossed with B6.129P2-LysZtm1(cre)J/cJ and backcrossed for 10 generations to the C57BL/6 background (Jackson Laboratory) and genotyped by PCR analysis of genomic DNA. Radiation chimeras were generated by exposing recipient mice to 2 doses of ionizing radiation (525 Gy) and reconstituting them with 2 x 10⁶ donor BM cells by injection into the tail vein. Hepatitis was induced by i.p. injection with 50 μg/kg E. coli 0111:B4 LPS (Sigma-Aldrich) plus 1 g/kg D-gal (Sigma-Aldrich) or by i.v. injection with 10 μg/kg TNF-α (R&D Systems) plus 1 g/kg GalN. When required, inhibitors were administrated by i.p. injection at 15 μg/kg as previously reported (50, 51). For in vivo neutrophil depletion, 300 μg/mouse of anti-Gr1 or the same volume of saline were i.v. injected 24 hours before challenge with LPS. Specificity of the depletion of cell populations was determined by flow cytometry of blood samples collected during the experiment.

Lentivirus vector production and infection of mice. Lentiviruses were produced as described (52). Transient calcium phosphate cotransfection of HEK-293 cells was done with the pGIZP empty vector or pGIZP.shEF2 vector (Thermo Scientific) together with pAd.B and pVSV-G. The supernatants containing the LV particles were collected, and a single-cell suspension was obtained and passed through a 40-μm filter. Viruses were collected by adding cold sterile PBS and were tittered by qPCR.

Mice were injected in the tail vein with 200 μl lentiviral particles suspended in PBS. Seven days after infection, mice were injected with LPS+D-gal.

Cell culture. Primary BM-derived macrophages were prepared and cultured as described previously (53). To isolate primary Kupffer cells from mouse livers, we performed collagenase perfusion and differential centrifugation using Percoll (Pharmacia) as described previously (54). BM neutrophils were purified by positive selection using biotinylated GR-1 (BD Biosciences – Pharmingen) and MACS streptavidin microbeads (Miltenyi Biotec). Neutrophil purity was 95% or greater, as assessed by flow cytometry (55). Cytokines in the culture medium were measured by multiplexed ELISA using a Luminex 200 analyzer (Millipore) and a mouse cytokine kit (Millipore). For primary hepatocyte isolation, liver parenchymal cells were prepared from 8- to 12-week-old anesthetized mice by in situ collagenase perfusion of the liver (56). Primary monolayer cultures were established by plating 1.5 x 10⁴ viable cells/cm² in 6-well collagen IV–coated plates. After 16 hours, primary hepatocytes were stimulated for 24 hours with TNF-α (20 ng/ml) plus CHX (100 μg/ml) or with 500 μl of macrophage-conditioned medium for 12 hours. Neutrophil migration assays were performed using BD Falcon FluoroBlok 96 Multi-Well insert systems. Neutrophils were stained for 1 hour with 1.5 μM calcine AM. Then, 1 x 10⁶ cells were plated well in the upper chamber in 50 μl of culture medium without FBS. Lower chambers contained 200 μl of culture medium alone or supplemented with the chemoattractant (20 ng/ml TNF-α or 1/10 cytokine cocktail). Fluorescence emission was measured at different time points with a fluorescent plate reader with bottom-reading capability (Fluoroskan Ascent; Thermo Scientific).

Serum analysis. Serum activities of ALT and AST were measured using the ALT and AST Reagent Kit (BioSystems Reagents) with a Benchmark Plus Microplate Spectrophotometer. Serum concentrations of cytokines were measured by multiplexed ELISA with a Luminex 200 analyzer (Millipore).

Biochemical analysis. Tissue extracts were prepared using Triton xylol buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 25 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and leupeptin). Extracts (30 μg protein) and immunoprecipitates (prepared from 2 mg protein) were examined by protein immunoblot analysis with antibodies to ERK1/2, phospho-ERK1/2, phospho-MKK3/6, p38α MAPK, phospho-p38, phospho-hsp27, phospho-JNK1/2, JNK1/2, caspase 3, cleaved caspase 3, cleaved PARP, and phospho-eEF2 kinase (Ser366), all from Cell Signaling and MKK3 (BD Biosciences), and GAPDH and eEF2 (Santa Cruz Biotechnology Inc.). The anti-p38γ and p38δ antibodies were raised against the peptides PRQRL-GAVPKETAL and PIARKDSRRSGMKL, respectively, which correspond to the C termini of the proteins.

Immunohistochemistry. Livers were fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Sections (5 μm) were stained with H&E. Isolation of liver-infiltrating mononuclear leukocytes. Mouse livers were collected, and a single-cell suspension was obtained and passed through a 70-μm strainer. Leukocytes were collected from the interphase of centrifuged Ficol gradients. Cells were counted with a CASY Cell Counter (57). Then 10⁶ cells were labeled by surface staining (PE-conjugated anti-CD11b and PerCP-Cy5.5-conjugated anti-Gr-1; Invitrogen), and another 10⁶ cells were collected, and a single-cell suspension was obtained and passed through a 70-μm strainer. Leukocytes were collected from the interphase of centrifuged Ficol gradients. Cells were counted with a CASY Cell Counter (57). Then 10⁶ Cells were labeled by surface staining (PE-conjugated anti-CD11b and PerCP-Cy5.5-conjugated anti-Gr-1; Invitrogen), and another 10⁶ cells were labeled in vitro with LPS (10 μg/ml) plus brefeldin A, which inhibits protein transport from ER to Golgi, leading to the accumulation of protein inside the ER (BD GolgiPlug) (1:1000) for 2 hours. The LPS-stimulated cells were then labeled by surface staining (PE-conjugated anti-CD11b and PerCP-conjugated anti-Gr-1; Invitrogen), fixed, permeabilized, and stained for intracellular TNF-α (APC-conjugated anti–TNF-α from BD Biosciences) and IL-12 (PE-conjugated anti–IL-12 from BD Biosciences). Flow cytometry was performed with a FACScan cytofluorometer (FACS Canto BD), and data were examined using FlowJo software.
TNF-α mRNA were determined by qRT-PCR. Relative mRNA levels were calculated by subtracting background from the level of the target mRNA and then using the standard ΔΔCt formula.

qRT-PCR. The expression of mRNA was examined by qRT-PCR using a 7900 Fast Real Time thermocycler and FAST SYBR GREEN assays (Applied Biosystems). Relative mRNA expression was normalized to Gapdh mRNA measured in each sample. Hif-1α, Gr-1, KC (Cxcl1), Mip-2 (Cxcl2), Mgp-1, and Icam-1 were amplified using the primers shown in Supplemental Table 1.

Statistics. Differences between groups were examined for statistical significance using 2-tailed Student’s t test or ANOVA coupled to Bonferroni’s post-test. Kaplan-Meier analysis of survival was performed using the log-rank test.

Study approval. Animal studies were approved by the local ethics committee and by the IACUC of the University of Massachusetts Medical School. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enacted under Spanish law 12/01/2005.

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Address correspondence to: Guadalupe Sabio, Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares Carlos III, C/ Melchor Fernández Almagro, 3, 28029 Madrid, Spain. Phone: 34.91453.12.00, ext. 2004; Fax: 34.91.453.12.45; E-mail: gsabio@cnic.es.